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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : <b>C12N 15/55, 9/80, 11/14, C12P 41/00, 13/04</b>		<b>A1</b>	(11) International Publication Number: <b>WO 00/23598</b>
			(43) International Publication Date: 27 April 2000 (27.04.00)
(21) International Application Number: <b>PCT/GB99/03458</b> (22) International Filing Date: 20 October 1999 (20.10.99) (30) Priority Data: 9822947.9                      20 October 1998 (20.10.98)      GB 9907739.8                      1 April 1999 (01.04.99)              GB (71) Applicant (for all designated States except US): <b>CHIROTECH TECHNOLOGY LIMITED [GB/GB];</b> Cambridge Science Park, Milton Road, Cambridge CB4 4WE (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): <b>TAYLOR, Stephen, John, Clifford [GB/GB];</b> Chirotech Technology Limited, Cambridge Science Park, Milton Road, Cambridge CB4 4WE (GB). <b>BROWN, Robert, Christopher [GB/GB];</b> Chirotech Technology Limited, Cambridge Science Park, Milton Road, Cambridge CB4 4WE (GB). (74) Agent: <b>GILL JENNINGS &amp; EVERY;</b> Broadgate House, 7 Eldon Street, London EC2M 7LH (GB).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>	
(54) Title: <b>AMINOACYLASE AND ITS USE IN THE PRODUCTION OF D-AMINOACIDS</b>			
(57) Abstract			
An isolated enzyme capable of hydrolysing N-acetyl-D-tryptophan at a substrate concentration of 10 g/l and which exhibits faster conversion of (R)-N-acetyl-2-thienylalanine than of (R)-N-acetyl-4-chlorophenylalanine. This enzyme is useful for preparing D-aminoacids.			

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AMINOACYLASE AND ITS USE  
IN THE PRODUCTION OF D-AMINOACIDS

Field of the Invention

This invention relates to an enzyme having D-aminoacylase activity and to its use  
5 in the production of D-aminoacids, by resolving a racemic mixture of N-acyl aminoacids  
and deprotecting optically-enriched N-acyl aminoacids.

Background of the Invention

D-Aminoacids are commercially important intermediates in the production of  
various pesticides, antibiotics and other pharmaceuticals. For example, phenylglycine and  
10 p-hydroxyphenylglycine are used in the synthesis of semi-synthetic penicillins and  
cephalosporins. There is also much demand for novel D-aminoacids as building blocks for  
new drug substances.

D-Aminoacids may be accessed by physical separation, for example by  
crystallisation of salts, or by asymmetric chemocatalysis by way of hydrogenation of an  
15 enamide precursor. Chemocatalysis provides a general method of broad applicability, e.g.  
for unnatural aminoacids, but requires subsequent N-deacylation for which conventional  
chemical hydrolysis often results in partial racemisation of the product. There are  
biocatalytic methods also, for example by the hydrolysis of hydantoins using a D-specific  
hydantoinase. However, the resulting D-carbamoyl aminoacid still requires enzymic or  
20 chemical deprotection to the aminoacid.

The production of L-aminoacids by means of an L-specific aminoacylase-catalysed  
hydrolysis of the racemic N-acetyl aminoacid is a technology that is well established. This  
uses the enzyme from *Aspergillus oryzae* and has been operated on a commercial basis at  
very large scale, to produce L-methionine, L-valine and L-phenylalanine. Such a large-  
25 scale technology does not exist for production of D-aminoacids, although D-aminoacylase  
activity has been identified in several microbial strains of *Pseudomonas*, *Streptomyces* and  
*Alcaligenes*. See Sugie and Suzuki, Agric. Biol. Chem. 44:1089-1095 (1980); Daicel  
Chemical Industries, JP 64-5488 (1989); Moriguchi and Ideta, Appl. Env. Microbiol. 54:  
2767-2770 (1988); Sakai *et al*, Agric. Biol. Chem. 54: 841-844 (1990); Sakai *et al*, J.  
30 Ferm. Bioeng. 71:79-82 (1991); Sakai *et al*, Appl. Env. Microbiol. 57: 2540-2543 (1991);  
Yang *et al*, Appl. Env. Microbiol. 57: 1259-1260 (1991); and Kameda *et al*, Nature 169:  
1016 (1952).

The enzymes from these strains were isolated and characterised. It should in theory be relatively easy to use such strains in whole-cell form for the resolution or deprotection of *N*-acetyl aminoacids, but the cells were also shown to contain L-aminoacylases, thus reducing the stereoselectivity. In addition, the low levels of activity, even after growth on inducing media, make purification and use of the enzyme from the whole-cell unattractive economically. A solution was foreseen by way of cloning the enzymes, and this has been reported recently for an *Alcaligenes* species D-aminoacylase, though it would not be expected that such an enzyme would work at any higher substrate concentration, nor differ significantly in its substrate specificity from the wild-type enzyme. See Moriguchi *et al*, Biosci. Biotech. Biochem. 57 (7):1149-1152 (1993); Wakayama *et al*, Biosci. Biotech. Biochem. 59 (11): 2115-2119 (1995); and Wakayama *et al*, Prot. Express. Pur. 7: 395-399 (1996).

This enzyme, obtained from *Alcaligenes xylosoxydans* subsp. *xylosoxydans* ("*Alcaligenes* A-6"), NCIMB 10771, does not hydrolyse N-acetyl-D-tryptophan. It is reported that the activity of D-aminoacylase is inhibited by 37% and 40% by D-phenylalanine and N-acetyl-D-alloisoleucine at a very low concentration of 2mM. This suggests that the enzyme is susceptible to severe product and substrate inhibition.

US-A-5206162 discloses a D-aminoacylase obtained from *Alcaligenes faecalis*, CCRC 14817.

EP-A-0896057 (published after the first priority date claimed for this Application) discloses a D-aminoacylase obtained from *Amycolatopsis orientalis*, IFO 12806.

#### Summary of the Invention

The present invention was made following a screen for D-aminoacylase activity performed on a collection of bacteria, and from this screen several were identified as having a D-aminoacylase. Five of these strains were used for genomic DNA preparation. It was then possible, by examining a known literature sequence, to design oligonucleotide primers, and use these in PCR experiments to generate a 1.4kb fragment possessing D-aminoacylase activity. The recombinant fragment was sub-cloned into pTrc99C expression vector. The recombinant plasmid carrying the D-amino acylase fragment was then transformed in to *E. coli* DH5 for over-expression. After fermentation of the host, cells were obtained which had good D-aminoacylase activity.

Sequencing of the enzyme showed that it had six differences to the published sequence of the known cloned *Alcaligenes* D-aminoacylase. These were Ser<sup>2</sup> to Ala; Gln<sup>3</sup> to Glu; Ala<sup>14</sup> to Val; Gly<sup>126</sup> to Arg; Gly<sup>240</sup> to Arg; and Glu<sup>242</sup> to Lys. These differences, individually or in combination, apparently bring about notable and surprising differences in the properties of the enzyme. For example, the novel enzyme will hydrolyse N-acetyl-D-tryptophan, whereas the published enzyme does not. Surprisingly, this enzyme is active at high substrate concentration; the published literature gives only examples of low substrate concentration, in the region of 20mM. At these concentrations, the volume efficiency is low, which increases the cost of recovering the product and reduces the economic viability of the process. Thus, it was surprising to find that the enzyme is effective at 100g/l of substrate; even at 200g/l good activity was demonstrated. It is useful at high volume efficiency, of about 100g/l, for the deprotection of several (D)-N-acetyl amino acids. This allows an economical process to be developed.

More generally, an isolated enzyme according to the present invention is capable of hydrolysing N-acetyl-D-tryptophan at a substrate concentration of 10 g/l. Thus, it is capable of the desired activity at the given concentration, and also at higher concentrations. In addition, unlike the enzymes disclosed in US-A-5206162 and in EP-A-0896057, it exhibits the ability to convert (R)-N-acetyl-2-thienylalanine, and also to convert it faster than (R)-N-acetyl-4-chlorophenylalanine.

## Description of the Invention

In general terms, the substrate used in the invention may be part of a mixture of the (L)- and (D)-N-acetyl amino acids. Alternatively, the (D)-N-acetyl amino acid may be enantiomerically enriched, e.g. essentially optically pure.

The novel enzyme may be used to produce natural and unnatural amino acids. One class of the latter is aryl/heteroaryl-substituted amino acids.

In some instances, particularly with substrates of a hydrophobic nature, the enzyme may suffer from substrate inhibition. In these cases, for example with N-acetyl-D-tryptophan or N-acetyl-D-2-naphthylalanine, a high substrate concentration may merely lead to a low conversion to product, so that a volume efficient reaction is not possible. However, this effect can be overcome by the simple expedient of adding the substrate in several batches over the course of the biotransformation, and, if kept at low concentration, a high product accumulation is possible. For example, if the enzyme is exposed to >20g/l

of N-acetyl-D-2-naphthylalanine, substrate hydrolysis is poor. However, the enzyme will hydrolyse 15 g/l efficiently and, by making several additions of the substrate, it is possible to accumulate about 75 g/l of D-2-naphthylalanine.

The enzyme may be used in whole cell or isolated form. It may be immobilised,  
5 if desired, by methods known to those of ordinary skill in the art.

The enzyme may be produced from the deposited organism (details given below).  
Alternatively, it may be produced by recombinant technology.

Using the DNA and amino-acid sequence provided herein, a person skilled in the art can readily construct fragments or mutations of the genes and enzymes disclosed  
10 herein. These fragments and mutations, which retain the activity of the exemplified enzyme, are within the scope of the present invention. Also, because of the redundancy of the genetic code, a variety of different DNA sequences can encode the amino-acid sequences disclosed herein. It is well within the skill of one of ordinary skill in the art to create these alternative DNA sequences encoding the same, or similar, enzymes. These  
15 DNA sequences are within the scope of the present invention. As used herein, reference to "essentially the same" sequence refers to sequences which have amino-acid substitutions, deletions, additions or insertions which do not materially affect activity. Fragments retaining activity are also included in this definition.

The genes of this invention can be isolated by known procedures and can be  
20 introduced into a wide variety of microbial hosts. Expression of the gene results, directly or indirectly, in the intracellular production and maintenance of the enzyme. The gene may be introduced *via* a suitable vector into a microbial host.

A wide variety of ways are available for introducing the gene into the microorganism host under conditions which allow for stable maintenance and expression  
25 of the gene. A DNA construct may include the transcriptional and translational regulatory signals for expression of the gene, the gene under their regulatory control and a DNA sequence homologous with a sequence in the host organism, whereby integration will occur, and/or a replication system which is functional in the host, whereby integration or stable maintenance will occur.

30 In the direction of transcription, namely in the 5' to 3' direction of the coding or sense sequence, the construct can involve the transcriptional regulatory region, if any, and the promoter, where the regulatory region may be either 5' or 3' of the promoter, the

ribosomal binding site, the initiation codon, the structural gene having an open reading frame in phase with the initiation codon, the stop codon(s), the polyadenylation signal sequence, if any, and the terminator region. This sequence as a double strand may be used by itself for transformation of a microorganism host, but will usually be included with a DNA sequence involving a marker.

The gene can be introduced between the transcriptional/translational initiation and termination regions, so as to be under the regulatory control of the initiation region. This construct can be included in a plasmid, which could include at least one replication system, but may include more than one, where one replication system is employed for cloning during the development of the plasmid and the second replication system is necessary for functioning in the ultimate host. In addition, one or more markers may be present, as described above. Where integration is desired, the plasmid will desirably include a sequence homologous with the host genome.

The transformants can be isolated in accordance with conventional ways, usually employing a selection technique, which allows for selection of the desired organism as against unmodified organisms or transferring organisms, when present. The transformants then can be tested for activity.

Suitable host cells include prokaryotes and eukaryotes. An example is *E. coli*.

The following Examples illustrate the invention.

#### 20 **Example 1**    **Production of D-Aminoacylase**

Genomic DNA was prepared from 5 *Alcaligenes* strains held in the Chirotech culture collection; CMC3352, 3353, 2916, 3378, 3823. From these genomic preparations PCR was carried out to amplify the D-aminoacylase reported by Wakayama *et al* (1995), supra. Primers were synthesised according to the published sequence of the *dam* gene from *Alcaligenes* A-6. The 5' PCR primer in SEQ ID NO. 1; the 3' PCR primer is SEQ ID NO. 2.

A 1.4 kb PCR fragment was amplified from strains CMC 3352 and 3353. These fragments were then cloned into the PCR cloning vector from Stratagene, pCR-script and transformed into *E. coli*. Resultant clones were analysed by restriction mapping to ascertain the presence of a 1.4kb acylase fragment. Clones harbouring this fragment were sequenced to verify that the putative acylase showed homology to the reported sequence. DNA sequence analysis show the majority of cloned fragments to include SEQ ID NO. 3.

The deduced aminoacid sequence is given below as SEQ ID NO. 4. The residues of the recombinant D-acylase differ from the published sequence as follows; Ser<sup>2</sup> to Ala; Gln<sup>3</sup> to Glu; Ala<sup>14</sup> to Val; ; Gly<sup>126</sup> to Arg; Gly<sup>240</sup> to Arg; Glu<sup>242</sup> to Lys.

The recombinant fragment was sub-cloned into pTrc99C expression vector via the  
5 5' *Nco*I and 3' *Bam*HI engineered restriction sites. The recombinant plasmid carrying the D-amino acylase fragment was transformed into *E. coli* DH5 for over-expression.

The recombinant cells, *E. coli* strain CMC 4406, have been deposited at NCIMB, 23 St. Machar Drive, Aberdeen AB24 3RY, Scotland. The accession number is NCIMB 40965.

10 The recombinant cells were grown by fermentation in a medium containing glucose, peptides and salts. The seed culture was inoculated from plates, and incubated overnight in TSB medium containing 0.1 g/l ampicillin at 37 °C.

The inoculum (5ml, OD 5.0) was grown in 1.5l of the following medium which contained (amounts in g.l<sup>-1</sup> unless otherwise indicated):

15	KH <sub>2</sub> PO <sub>4</sub>	8
	K <sub>2</sub> HPO <sub>4</sub>	7
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1
	MgSO <sub>4</sub> 7H <sub>2</sub> O	1
	Yeast Extract	15
20	Trace elements solution	1ml.l <sup>-1</sup>
	Glucose	10
	Polypropylene glycol	1ml.l <sup>-1</sup>
	Hycase SF	15

25 The trace elements solution consisted of (amounts in g.l<sup>-1</sup> unless otherwise indicated):

	CaCl <sub>2</sub> .2H <sub>2</sub> O	3.6
	CoCl <sub>2</sub> . 6H <sub>2</sub> O	2.4
	CuCl <sub>2</sub> . 2H <sub>2</sub> O	0.85
	FeCl <sub>3</sub> . 6H <sub>2</sub> O	5.4
30	H <sub>3</sub> BO <sub>4</sub>	0.3
	HCl	333ml.l <sup>-1</sup>
	MnCl <sub>2</sub> . 4H <sub>2</sub> O	2.0
	Na <sub>2</sub> MoO <sub>4</sub> . 2H <sub>2</sub> O	4.8
35	ZnO	2.0



The pH was controlled between 6.9 and 7.2 by the addition of NaOH solution, and the temperature maintained at 30°C. IPTG (0.24g/l) was added after inoculation. After 24 hrs, the biomass had reached an OD of 34. Cells were then harvested by centrifugation and stored at -15°C, then used in biotransformations as required.

5    **Example 2**    **Deprotection of (D)-N-Acetyl-(1-bromovinyl)alanine**

- $\text{KH}_2\text{PO}_4$  (0.8g, 10mmol) was dissolved in water (800 ml) in a 2 litre jacketed vessel. (D)-N-acetyl-(1-bromovinyl)alanine (100g, 0.42 mol, ~95% ee<sub>R</sub>) was added and the pH adjusted to 8.0 using NaOH (46-48%). The temperature of the jacketed vessel was raised to 40°C and the solution stirred for 10 minutes while maintaining the pH at 8. The
- 10    D-aminoacylase enzyme whole cells (9g) were added in one portion and the reaction mixture stirred at 40°C while maintaining the pH at 8.0 by subsequent additions of NaOH. The reaction was monitored by chiral GC as follows: 0.5 ml of the reaction mixture was taken and acidified to pH 2.0 with conc. HCl. The aqueous was extracted with EtOAc which was dried ( $\text{MgSO}_4$ ) and filtered and treated with 0.1ml of TMS-diazomethane. The
- 15    derivatised product was assayed by chiral GC (Chrompack Chirasil L-Val, 25m, 20psi He, 60°C for 10 mins, 5°C/min to 200°C, holding for 10 minutes, FID detection). After 1 hour the ee of the substrate had decreased to 68%, after 2 hours it was 24% and after 22 hours was 7%. The reaction mixture was then acidified to pH 2.0 using concentrated HCl solution, then filtered through a celite pad, and washed with EtOAc (3 x 300ml).
- 20    The solution was then adjusted to pH 6.5 using NaOH (46-48%) and concentrated under reduced pressure until about 200 ml of solution remained. A white solid crashed out of solution and was filtered off and washed with acetone. This gave the product (D)-(1-bromovinyl)alanine as a clean white solid (60g, ee<sub>R</sub>>99 %). The enantiomeric excess was measured by HPLC (25cm Chirobiotic T column, 70:30 MeOH:water, 1ml/min,
- 25    210nm). Other aminoacids were determined by the same method or with variations in the MeOH:water mobile phase composition.

**Example 3**    **Deprotection of (D)-N-acetylpropargylglycine**

- $\text{KH}_2\text{PO}_4$  (2.1g) was dissolved in water (2.5l) in a 2 litre jacketed vessel. The (D)-N-acetylpropargylglycine (232g, 1.50mol, ~95% ee<sub>R</sub>) was added and the pH adjusted to
- 30    8.0 using NaOH (46-48%). The temperature of the jacketed vessel was raised to 40°C and the solution stirred for 10 minutes while maintaining the pH at 8. The D-aminoacylase enzyme whole cells (7g) were added in one portion and the reaction mixture stirred at

40°C while maintaining the pH at 8.0 by subsequent additions of NaOH. After 88 hours the remaining  $ee_{(R)-N-Ac}$  was 25.8%, only marginally lower than that at 16 hours. Another 3%/wt of cells were added and after 104 hrs the  $ee_{(R)-N-Ac}$  was 17.8%. The biotransformation was worked up since assuming 99% ee of product conversion was 85%.

- 5            Work-up and isolation as for Example 2 gave a brownish solid (271g) which was slurried in MeOH for 10 minutes to give a clean white solid (171g) of >99% ee (R)-propargylglycine.

**Example 4    Deprotection of (D)-N-acetyl-2-furylalanine**

- (D)-N-Acetyl-2-furylalanine (18g,  $ee_R > 99\%$ ) was added to water (200ml) containing 10mmole of  $KH_2PO_4$  and the temperature of the jacketed vessel raised to 40°C. The suspension was then adjusted to pH 8.0 with NaOH and stirred for 10 minutes. 0.72g (4% wt) of D-aminoacylase whole cells were then added and the reaction stirred vigorously while maintaining the pH at 8.0 by subsequent addition of NaOH. The reaction was followed by chiral GC by the method described above, and was complete after 2 hours. The aqueous was then acidified to pH 2.0 with conc. HCl, and filtered through a Celite pad. The filtrate was then washed with EtOAc and adjusted to pH 7 with NaOH. The solution was then treated with  $Na_2CO_3$  (2 equivalents assuming 100% conversion), cooled to ~10°C and then a solution of Fmoc-OSu (1 equivalent) in THF (300ml) added. After work-up the product was isolated as a white solid and recrystallised from methanol/water to give 18.4g of >99% ee (D)-2-furylalanine.
- 10  
15  
20

**Example 5    Deprotection of (D)-N-acetylallylglycine**

- (D)-N-Acetylallylglycine (40g,  $ee_R$  89%) was added to water (200ml) containing 10mmole of  $KH_2PO_4$  and the temperature of the jacketed vessel raised to 40°C. The suspension was then adjusted to pH 8.0 with NaOH and stirred for 10 minutes. 1.0g (2.5% wt) of D-aminoacylase whole cells were then added and the reaction stirred vigorously while maintaining the pH at 8.0 by subsequent addition of NaOH. After 3 hours HPLC showed 30% conversion. After 16 hours the reaction had not gone any further and a further 2.5% wt of whole cells were added. After 60 hours the reaction still had not progressed past 30% and the solution was diluted to 10% by the addition of water. After another 2 hours, the reaction had reached 44% conversion, and after 88 hours 82% conversion.
- 25  
30

**Example 6 Deprotection of (D)-N-acetyl-2-naphthylalanine**

(D)-N-Acetyl-2-naphthylalanine (0.75g) was added to 50ml Tris buffer (0.1 M, pH 7.5, 30 °C). The suspension was then adjusted to pH 8.0 with NaOH and stirred for 10 minutes. A crude lysate of D-aminoacylase (3 ml, 165U, 1U=hydrolysis of 1 $\mu$ mol of N-acetyl-D-tryptophan/min at 25°C, pH 7.5, 0.1M Tris buffer) was then added and the reaction stirred while maintaining the pH at 8.0 by subsequent addition of NaOH. More substrate (0.75g) and enzyme (165U) was added at 22, 46, 96, 173 and 218 hours. The final conversion by HPLC was 95% by peak area.

**Example 7 Deprotection of (D)-N-acetyl-3-pyridylalanine**

(D)-N-Acetyl-3-pyridylalanine (50g) was added to 750ml Tris buffer (0.1 M, pH 7.5, 30°C). The suspension was then adjusted to pH 8.0 with NaOH and stirred for 10 minutes. A crude lysate of D-aminoacylase (20 ml, 1100U, 1U=hydrolysis of 1 $\mu$ mol of N-acetyl-D-tryptophan/min at 25°C, pH 7.5, 0.1M Tris buffer) was then added and the reaction stirred while maintaining the pH at 8.0 by subsequent addition of NaOH. After 15 hours the reaction had reached about 80% conversion as measured by HPLC peak area.

**Examples 8 to 20 D-Acylase Reactions**

Table 1 reports D-acylase reactions using a range of unnatural (R)-N-Ac-phenylalanine and (R)-N-Ac-alanine derivatives, and (R)-N-Ac-4-fluorophenylglycine.

**Table 1**

20

Ex.	Substrate (N-Acetyl-)	Substrate Conc.		Enzyme (U/mmol)	pH <sup>b</sup>	Temp (°C)	Time <sup>c</sup>	Yield <sup>d</sup> (%)	Conv <sup>e</sup> (%)
		g/L	Mol/L						
8	4-Chloro-Phe-Ala	20	0.083	1.0 <sup>g</sup>	8.0	40	72hrs	67	61
9	4-Bromo-Phe-Ala	30	0.104	50.2	8.6	40	>3days	73	90
10	4-Fluoro-Phe-Ala	84	0.373	26.5	7.5	30	4days	73	98
11	4-Cyano-Phe-Ala	15	0.069	18.5	7.7-8.0	30	>3days	45	75
12	2-Fluoro-Phe-Ala	100	0.440	2.2	8.0	40	24hrs	79	90
13	Styryl-Ala	13	0.056	160	8.5	30	3days	84	90
14	2-Thienyl-Ala	70	0.330	21.3	7.7	30	3days	56	95
15	5-Br-2-thienyl-Ala	44.4	0.152	14.6 <sup>g</sup>	8.2	40	48hrs	86	>95
16	3-Thienyl-Ala	100	0.470	24.3	8.5	30	48hrs	87	94
17	3-Furyl-Ala	30	0.152	105	8.5	40	24hrs	n/a	90
18	2-Naphthyl-Ala	15	0.062	3.80 <sup>g</sup>	8.5	40	7days	n/a	90
19	TAZ	112	0.525	69.1	7.5	30	3days	n/a	90
20	4-Fluoro-Phe-Gly	85.7	0.406	21.6	8.1	35	24hrs	88	n/a

<sup>b</sup> All reactions carried out in NaOH/KH<sub>2</sub>PO<sub>4</sub> buffer. <sup>c</sup> Time to reach completion/cessation of reaction. <sup>d</sup> Isolated yield. <sup>e</sup> Conversion in crude biotransformation reaction mixture. <sup>f</sup>%e.e. rose from 90% in N-acetyl substrate to 96.5% in aminoacid. <sup>g</sup> Reaction done using whole cells.

5 TAZ = 4-ThiazoylAlanine

### Comparative Testing

In order to evaluate the properties of the novel enzyme and known D-acylases, comparative experiments were run. The results are reported in Table 2. In each set of 3 results, the respective enzymes were those deposited as IFO 12806 (104470) and CCRC  
10 14817 (104476), and that of Example 1 (D-Ace). The results (U/U) show that, when appropriate corrections have been made, the novel enzyme converts certain unnatural aminoacids, e.g. (R)-N-Ac-thienylalanine, faster than CCRC 14817, although the rate is slower for (R)-N-Ac-4-chlorophenylalanine.

### Example 21 Whole Cell Immobilisation

15 Whole cells of *E. coli* CMC4406 containing recombinant D-acylase were immobilised on a reactive soluble polymer (RSP). The RSP was prepared by reaction of polyethyleneimine (0.8g) with aqueous 25% w/v glutaraldehyde (1.6ml), to a total volume of 20 ml H<sub>2</sub>O. The RSP was then mixed with 10g of cells resuspended in 20ml H<sub>2</sub>O. This was stirred vigorously for 30 minutes, after which the immobilised cells, having the  
20 consistency of foam rubber, were recovered by filtration. The final product (20g) had a specific activity of 20.55 U/g and the recovery of activity was 43% of the whole cells used in the immobilisation. 1 Unit of activity is defined as the hydrolysis of 1µmol/min N-Ac-D-tryptophan to D-tryptophan measured at a substrate concentration of 10mM at 25°C, pH7.5.

### 25 Example 22 Whole Cell Immobilisation

An aqueous suspension of cells of *E. coli* CMC4406 (10g in 20ml of water) was mixed thoroughly with 0.8g PEI, before the addition of 1.6ml of 25% w/v glutaraldehyde. Stirring of this mixture resulted in the formation of bead-like aggregates which were recovered by filtration. The final product (13.5g) had a specific activity of 20.39U/g and  
30 yielded 25% of the starting activity.

Table 2

Enz loading									
Substrate	Enzyme	Dilution	U/ml	Sub Loading	%	Time	Activity	Activity	Activity
			(N-Ac-DL-Trp)	(mM)	Conversion	(Hrs)	(U/U)	(U/g)	(U/ml)
(+/-)-N-Ac-4-Fluoro-Phe-Gly	104470	2	0.005	10	2	30	2	0	0
(+/-)-N-Ac-4-Fluoro-Phe-Gly	104476	2	0.115	10	22.4	30	1	2	0
(+/-)-N-Ac-4-Fluoro-Phe-Gly	D-Ace	200	0.2775	10	7.6	30	0	20	8
(R)-N-Ac-Thienyl Ala	104470	2	0.005	10	0.3	30	-	-	-
(R)-N-Ac-Thienyl Ala	104476	2	0.115	10	26.6	30	1	3	0
(R)-N-Ac-Thienyl Ala	D-Ace	200	0.2775	10	65	6	7	840	361
(R)-N-Ac-Naphthyl Ala	104470	2	0.005	50	1.5	22	11	1	0
(R)-N-Ac-Naphthyl Ala	104476	2	0.115	50	2.4	22	1	2	0
(R)-N-Ac-Naphthyl Ala	D-Ace	200	0.2775	50	1.7	22	0	30	13
(R)-N-Ac-Styryl Ala	104470	2	0.005	50	1.8	22	14	1	0
(R)-N-Ac-Styryl Ala	104476	2	0.115	50	1	22	-	-	-
(R)-N-Ac-Styryl Ala	D-Ace	200	0.2775	50	3.5	22	0	62	27
(R)-N-Ac-4-Chloro-Phe-Ala	104470	2	0.005	50	0.3	22	-	-	-
(R)-N-Ac-4-Chloro-Phe-Ala	104476	2	0.115	50	7.1	22	2	5	1
(R)-N-Ac-4-Chloro-Phe-Ala	D-Ace	200	0.2775	50	26	22	4	458	197
N-Ac-DL-Leu	104470	2	0.005	20	29	4	483	48	5
N-Ac-DL-Leu	104476	10	0.023	20	31	4	112	258	26
N-Ac-DL-Leu	D-Ace	5000	0.0111	20	13	4	98	12,597	5,417
N-Ac-DL-Met	104470	2	0.005	20	21	4	350	35	4
N-Ac-DL-Met	104476	10	0.023	20	17	4	62	142	14
N-Ac-DL-Met	D-Ace	5000	0.0111	20	7	4	53	6,873	2,917

Table 2 (contd.)

N-Ac-DL-Phe	104470	10	0.001	20	5	5.25	317	32	3
N-Ac-DL-Phe	104476	20	0.0115	20	22	5.25	121	279	28
N-Ac-DL-Phe	D-Ace	5000	0.0111	20	6	5.25	34	4,430	1,905
N-Ac-DL-Trp	104470	2	0.005	25	16	23.5	57	6	1
N-Ac-DL-Trp	104476	2	0.115	25	36	1	130	300	30
N-Ac-DL-Trp	D-Ace	5000	0.0111	25	13	2	244	31,492	13,542
N-Ac-DL-Val	104470	10	0.001	20	9	4	750	75	8
N-Ac-DL-Val	104476	10	0.023	20	38	4	138	317	32
N-Ac-DL-Val	D-Ace	1000	0.0555	20	4	4	6	775	333
N-Ac-D-Phe	104470	10	0.001	10	7	5.25	222	22	2
N-Ac-D-Phe	104476	20	0.0115	10	14	5.25	39	89	9
N-Ac-D-Phe	D-Ace	5000	0.0111	10	10	1	150	19,380	8,333
N-Ac-D-Trp	104470	2	0.005	10	20	23.5	28	3	0
N-Ac-D-Trp	104476	2	0.115	10	38	2	26	60	6
N-Ac-D-Trp	D-Ace	5000	0.0111	10	14	1	210	27,132	11,667
N-Benzoyl-DL-Phe	104470	10	0.001	20	0.1	5.25	-	-	-
N-Benzoyl-DL-Phe	104476	10	0.023	20	42	5.25	116	267	27
N-Benzoyl-DL-Phe	D-Ace	5000	0.0111	20	1.7	5.25	10	1,255	540

CLAIMS

1. An isolated enzyme capable of hydrolysing N-acetyl-D-tryptophan at a substrate concentration of 10 g/l and which exhibits faster conversion of (R)-N-acetyl-2-thienylalanine than of (R)-N-acetyl-4-chlorophenylalanine.
- 5 2. An isolated enzyme having the aminoacid sequence of SEQ ID NO: 4, or a fragment thereof capable of hydrolysing N-acetyl-D-tryptophan at a substrate concentration of 10 g/l.
3. An enzyme according to claim 1 or claim 2, wherein the substrate concentration is 30 g/l.
- 10 4. An enzyme according to claim 3, wherein the substrate concentration is 100 g/l.
5. An enzyme according to any preceding claim, in immobilised form.
6. An isolated polynucleotide encoding an enzyme according to claim 2.
7. A polynucleotide according to claim 6, having part or all of the sequence shown in SEQ ID No. 3.
- 15 8. A microorganism transformed to express an enzyme according to any of claims 1 to 5.
9. A microorganism having the characteristics of NCIMB 40965.
10. A method for producing an enzyme according to any of claims 1 to 5, which comprises culturing a microorganism according to claim 8 or claim 9.
- 20 11. A process for the preparation of a (D)-aminoacid, which comprises the conversion of a corresponding (D)-N-acylaminoacid using an enzyme according to any of claims 1 to 5 or a microorganism according to claim 8 or claim 9.
12. A process according to claim 11, wherein the concentration of the N-acylaminoacid is at least 30g/l.
- 25 13. A process according to claim 11, wherein the concentration of the N-acylaminoacid is at least 100 g/l.
14. A process according to any of claims 11 to 13, wherein the (D)-N-acylaminoacid is part of a mixture of the (L)- and (D)-N-acylaminoacids.
15. A process according to any of claims 11 to 13, wherein the (D)-N-acylaminoacid is enantiomerically enriched.
- 30 16. A process according to any of claims 11 to 13, wherein the (D)-N-aminoacid is essentially a single enantiomer.

17. A process according to claims 11 to 16, wherein the aminoacid is unnatural.
18. A process according to any of claims 11 to 15, wherein the substrate is hydrophobic, such that there is substrate inhibition, which comprises adding the substrate batchwise during the conversion.
- 5 19. A process according to any of claims 11 to 18, wherein the concentration of the accumulated D-aminoacid is at least 30 g/l.



## SEQUENCE LISTING

<110> Chirotech Technology Limited

<120> AMINOACYLASE AND ITS USE IN THE PRODUCTION OF  
D-AMINOACIDS

<130> REP05858WO

<140> not yet known

<141> 1999-10-20

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ctg ggc gtg cgc ggc gac cgc atc gcc gcc atc ggc gat ctg tcg gac      144
Leu Gly Val Arg Gly Asp Arg Ile Ala Ala Ile Gly Asp Leu Ser Asp
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cgc gac atg acg ccc aag atc tcg cag ggc gtc acc acg gtg gtc acg      288
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ggc aat tgc ggc atc agc ctg gca ccg ctg gcg cac gcc aac ccg ccc      336
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ttc gcc gac tac ctg gac gcg ttg cgg gcc acg ccg gcg gcc gtc aac      432
Phe Ala Asp Tyr Leu Asp Ala Leu Arg Ala Thr Pro Ala Ala Val Asn
            130              135              140

gcc gcc tgc atg gtg ggc cat tcg acg ctg cgc gcc gcg gtc atg ccg      480
Ala Ala Cys Met Val Gly His Ser Thr Leu Arg Ala Ala Val Met Pro
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Asp Leu Gln Arg Ala Ala Thr Asp Glu Glu Ile Ala Ala Met Arg Asp
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&lt;212&gt; PRT

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&lt;400&gt; 4

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Val Arg Gly Asp Arg Ile Ala Ala Ile Gly Asp Leu Ser Asp Ala Ala  
 35 40 45

Ala His Thr Arg Val Asp Val Ser Gly Leu Val Val Ala Pro Gly Phe  
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Ile Asp Ser His Thr His Asp Asp Asn Tyr Leu Leu Arg Arg Arg Asp  
 65 70 75 80

Met Thr Pro Lys Ile Ser Gln Gly Val Thr Thr Val Val Thr Gly Asn  
 85 90 95

Cys Gly Ile Ser Leu Ala Pro Leu Ala His Ala Asn Pro Pro Ala Pro  
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Leu Asp Leu Leu Asp Glu Gly Gly Ser Tyr Arg Phe Glu Arg Phe Ala  
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Asp Tyr Leu Asp Ala Leu Arg Ala Thr Pro Ala Ala Val Asn Ala Ala  
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Cys Met Val Gly His Ser Thr Leu Arg Ala Ala Val Met Pro Asp Leu  
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Tyr Pro Pro Ala Ala Arg Ala Thr Thr Glu Glu Ile Ile Glu Val Cys  
 195 200 205

Arg Pro Leu Ser Ala His Gly Gly Ile Tyr Ala Thr His Met Arg Asp  
 210 215 220

Glu Arg Glu His Ile Val Ala Ala Leu Glu Glu Thr Phe Arg Ile Arg  
 225 230 235 240

Arg Lys Leu Asp Val Pro Val Val Ile Ser His His Lys Val Met Gly  
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Gln Pro Asn Phe Gly Arg Ser Arg Glu Thr Leu Pro Leu Ile Glu Ala  
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Ala Met Ala Arg Gln Asp Val Ser Leu Asp Ala Tyr Pro Tyr Val Ala  
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Gly Ser Thr Met Leu Lys Gln Asp Arg Val Leu Leu Ala Gly Arg Thr  
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Ile Ile Thr Trp Cys Lys Pro Phe Pro Glu Leu Ser Gly Arg Asp Leu  
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# INTERNATIONAL SEARCH REPORT

Int. Application No  
PCT/GB 99/03458

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 7 C12N15/55 C12N9/80 C12N11/14 C12P41/00 C12P13/04		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N C12P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>M. WAKAYAMA ET AL.: "Cloning and sequencing of a gene encoding D-aminoacylase from <i>Alcaligenes xylosoxydans</i> subsp. <i>xylosoxydans</i> A-6 and expression of the gene in <i>Escherichia coli</i>."</p> <p>BIOSCIENCE, BIOTECHNOLOGY AND BIOCHEMISTRY, vol. 59, November 1995 (1995-11), pages 2115-2119, XP002105914 cited in the application the whole document</p> <p style="text-align: center;">— — — — — — / —</p>	1-19
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>		
* Special categories of cited documents :		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search  <div style="text-align: center; font-weight: bold;">17 December 1999</div>		Date of mailing of the international search report  <div style="text-align: center; font-weight: bold;">11/01/2000</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016		Authorized officer  <div style="text-align: center; font-weight: bold;">Hix, R</div>

## INTERNATIONAL SEARCH REPORT

Int. .ional Application No

PCT/GB 99/03458

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	M. MORIGUCHI ET AL.: "Production, purification and characterization of D-aminoacylase from Alcaligenes xylosoxydans subsp. xylosoxydans A-6" BIOSCIENCE, BIOTECHNOLOGY AND BIOCHEMISTRY, vol. 57, July 1993 (1993-07), pages 1149-1152, XP002105915 cited in the application the whole document	1-19
Y	US 5 206 162 A (TSAI YING C ET AL) 27 April 1993 (1993-04-27) the whole document	1-19
Y,P	EP 0 896 057 A (DAICEL CHEM) 10 February 1999 (1999-02-10) the whole document	1-19
Y	M. WAKAYAMA ET AL.: "Overproduction of D-aminoacylase from Alcaligenes xylosoxydans subsp. xylosoxydans A-6 in Escherichia coli and its purification." PROTEIN EXPRESSION AND PURIFICATION, vol. 7, 1996, pages 395-399, XP002105916 cited in the application the whole document	1-19
A	M. SUGIE ET AL.: "Optical resolution of DL-amino acids with D-aminoacylase of Streptomyces." AGRICULTURAL & BIOLOGICAL CHEMISTRY, vol. 44, no. 5, 1980, pages 1089-1095, XP002105917 cited in the application the whole document	
A	EP 0 304 021 A (TAKEDA CHEMICAL INDUSTRIES LTD) 22 February 1989 (1989-02-22) table 3	
A	DE 24 19 838 A (AJINOMOTO KK) 21 November 1974 (1974-11-21) example 4	
A	DE 23 44 060 A (AJINOMOTO KK) 7 March 1974 (1974-03-07) example 4	
A	PATENT ABSTRACTS OF JAPAN vol. 014, no. 548 (C-0785), 5 December 1990 (1990-12-05) & JP 02 234677 A (DAI ICHI PURE CHEM CO LTD), 17 September 1990 (1990-09-17) abstract	
	— -/-	



# INTERNATIONAL SEARCH REPORT

Int. J. Application No

PCT/GB 99/03458

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PATENT ABSTRACTS OF JAPAN vol. 013, no. 174 (C-589), 25 April 1989 (1989-04-25) &amp; JP 01 005488 A (DAICEL CHEM IND LTD), 10 January 1989 (1989-01-10) abstract</p>	
A	<p>PATENT ABSTRACTS OF JAPAN vol. 011, no. 353 (C-457), 18 November 1987 (1987-11-18) &amp; JP 62 126976 A (AGENCY OF IND SCIENCE &amp; TECHNOL;OTHERS: 02), 9 June 1987 (1987-06-09) abstract</p>	

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/03458

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US 5206162	A	27-04-1993	NONE		
EP 0896057	A	10-02-1999	JP 11098982	A	13-04-1999
			US 5916774	A	29-06-1999
EP 0304021	A	22-02-1989	AT 88753	T	15-05-1993
			CN 1035320	A, B	06-09-1989
			DE 3880585	A	03-06-1993
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			JP 1137973	A	30-05-1989
			JP 2712331	B	10-02-1998
			KR 9700185	B	06-01-1997
			US 4981799	A	01-01-1991
DE 2419838	A	21-11-1974	JP 907281	C	08-05-1978
			JP 49132293	A	18-12-1974
			JP 52033194	B	26-08-1977
			GB 1422919	A	28-01-1976
			NL 7405450	A	28-10-1974
			US 3907638	A	23-09-1975
DE 2344060	A	07-03-1974	JP 824490	C	31-07-1976
			JP 49042887	A	22-04-1974
			JP 50037277	B	01-12-1975
			CA 1018920	A	11-10-1977
			CH 577438	A	15-07-1976
			FR 2197836	A	29-03-1974
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JP 02234677	A	17-09-1990	JP 2869793	B	10-03-1999
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			JP 7083711	B	13-09-1995
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## SEQUENCE LISTING

<110> Chirotech Technology Limited

<120> AMINOACYLASE AND ITS USE IN THE PRODUCTION OF  
D-AMINOACIDS

<130> REF05058WO

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Ala Ala Ala His Thr Arg Val Asp Val Ser Gly Leu Val Val Ala Pro
      50              55              60

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Gly Phe Ile Asp Ser His Thr His Asp Asp Asn Tyr Leu Leu Arg Arg
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cgc gac atg acg ccc aag atc tgg cag gcc gtc acc acg gtg gtc acc 288
Arg Asp Met Thr Pro Lys Ile Ser Gln Gly Val Thr Thr Val Val Thr
      80              85              90

ggc aat tgc gcc atc agc ctg gca ccg ctg gcc cac gcc aac ccg ccc 336
Gly Asn Cys Gly Ile Ser Leu Ala Pro Leu Ala His Ala Asn Pro Pro
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Phe Ala Asp Tyr Leu Asp Ala Leu Arg Ala Thr Pro Ala Ala Val Asn
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Ala Ala Cys Met Val Gly His Ser Thr Leu Arg Ala Ala Val Met Pro
      145             150             155

gac ttg cag cgc gcc gcc acc gac gag gaa atc gcc gcc atg cgg gac 528
Asp Leu Gln Arg Ala Ala Thr Asp Glu Glu Ile Ala Ala Met Arg Asp
      160             165             170

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Leu Ala Glu Glu Ala Met Ala Ser Gly Ala Ile Gly Ile Ser Thr Gly	
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gcc ttc tac ccg ccc gcc gcc cgc gcc acc acc gaa gag atc atc gag	624
Ala Phe Tyr Pro Pro Ala Ala Arg Ala Thr Thr Glu Glu Ile Ile Glu	
195 200 205	
gtg tgc cgg ccg ctg agc gcg cat ggc gcc atc tac gcc acc cac atg	672
Val Cys Arg Pro Leu Ser Ala His Gly Gly Ile Tyr Ala Thr His Met	
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cgc gac gaa cgc gag cac atc gtg gcc gcg ctg gag gaa acc ttc cgc	720
Arg Asp Glu Arg Glu His Ile Val Ala Ala Leu Glu Glu Thr Phe Arg	
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Ile Arg Arg Lys Leu Asp Val Pro Val Val Ile Ser His His Lys Val	
240 245 250	
atg gcc cag ccc aat ttc gcc cgc tgg cgc gag acc ctg ccg ctg atc	816
Met Gly Gln Pro Asn Phe Gly Arg Ser Arg Glu Thr Leu Pro Leu Ile	
255 260 265 270	
gag gcc gcc atg gcg cgc cag gac gtc tgg ctg gac gcg tat ccc tac	864
Gln Ala Ala Met Ala Arg Gln Asp Val Ser Leu Asp Ala Tyr Pro Tyr	
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gtg gcc gcc tcc acc atg etc aag cag gac cgc gtg ctg ctg gcc ggc	912
Val Ala Gly Ser Thr Met Leu Lys Gln Asp Arg Val Leu Leu Ala Gly	
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Arg Thr Ile Ile Thr Trp Cys Lys Pro Phe Pro Glu Leu Ser Gly Arg	
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Asp Leu Asp Glu Val Ala Ala Glu Arg Gly Lys Ser Lys Tyr Asp Val	
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Val Pro Glu Leu Gln Pro Ala Gly Ala Ile Tyr Phe Met Met Asp Glu	
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ccc gac gtg cag cgc atc ctg gcg ttc gcc ccg acc atg atc gcc tcc	1104
Pro Asp Val Gln Arg Ile Leu Ala Phe Gly Pro Thr Met Ile Gly Ser	
355 360 365	

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 Asp Gly Leu Pro His Asp Glu Arg Pro His Pro Arg Leu Trp Gly Thr  
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 Phe Pro Arg Val Leu Gly His Tyr Ala Arg Asp Leu Gly Leu Phe Pro  
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ctg gag acg gcg gta tgg aag atg acc ggc ctg acc gcc gcg cgc ttc 1248  
 Leu Glu Thr Ala Val Trp Lys Met Thr Gly Leu Thr Ala Ala Arg Phe  
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 Gly Leu Ala Gly Arg Gly Gln Leu Gln Ala Gly Tyr Phe Ala Asp Leu  
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 Val Val Phe Asp Pro Ala Thr Val Ala Asp Thr Ala Thr Phe Glu His  
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cat acc gag cgc gcc gcc ggc atc cat tcc gtg tac gtc aac ggc gcg 1392  
 Pro Thr Glu Arg Ala Ala Gly Ile His Ser Val Tyr Val Asn Gly Ala  
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Ala His Thr Arg Val Asp Val Ser Gly Leu Val Val Ala Pro Gly Phe  
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Ile Asp Ser His Thr His Asp Asp Asn Tyr Leu Leu Arg Arg Arg Asp  
 65 70 75 80

Met Thr Pro Lys Ile Ser Gln Gly Val Thr Thr Val Val Thr Gly Asn  
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Cys Gly Ile Ser Leu Ala Pro Leu Ala His Ala Asn Pro Pro Ala Pro  
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Asp Tyr Leu Asp Ala Leu Arg Ala Thr Pro Ala Ala Val Asn Ala Ala  
 130 135 140

Cys Met Val Gly His Ser Thr Leu Arg Ala Ala Val Met Pro Asp Leu  
 145 150 155 160

Gln Arg Ala Ala Thr Asp Glu Glu Ile Ala Ala Met Arg Asp Leu Ala  
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Glu Glu Ala Met Ala Ser Gly Ala Ile Gly Ile Ser Thr Gly Ala Phe  
 180 185 190

Tyr Pro Pro Ala Ala Arg Ala Thr Thr Glu Glu Ile Ile Glu Val Cys  
 195 200 205

Arg Pro Leu Ser Ala His Gly Gly Ile Tyr Ala Thr His Met Arg Asp  
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Glu Arg Glu His Ile Val Ala Ala Leu Glu Glu Thr Phe Arg Ile Arg  
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Arg Lys Leu Asp Val Pro Val Val Ile Ser His His Lys Val Met Gly  
 245 250 255

Gln Pro Asn Phe Gly Arg Ser Arg Glu Thr Leu Pro Leu Ile Glu Ala  
 260 265 270

Ala Met Ala Arg Gln Asp Val Ser Leu Asp Ala Tyr Pro Tyr Val Ala  
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Gly Ser Thr Met Leu Lys Gln Asp Arg Val Leu Leu Ala Gly Arg Thr  
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Ile Ile Thr Trp Cys Lys Pro Phe Pro Glu Leu Ser Gly Arg Asp Leu  
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